Materials and Methods

Patient Enrollment

In this study, we enrolled patients with sporadic thoracic AAD who were undergoing elective open operation to replace the diseased aorta with a graft. During aneurysm repair, we routinely excised tissue from the anterior-lateral portion of the aortic wall (the outer wall of the false lumen in dissection cases) in the region of the largest aortic diameter; a portion of this discarded tissue was collected for study. Patient characteristics are shown in Table 1. In addition, we used aortic tissues from age-matched organ donors without aortic aneurysm, dissection, coarctation, or previous aortic repair as control samples (International Institute for the Advancement of Medicine, Jessup, PA). To minimize aortic damage due to poor circulation, we selected donors with cardiac arrest for less than 60 minutes. The aortas were collected within 60 minutes of termination of life support. They were preserved in UW Belzer solution and shipped to our lab on wet ice. The time from aorta collection to tissue processing/banking was less than 24 hours. Periaortic fat and intraluminal thrombus were trimmed from all aortic samples. The samples were rinsed with 0.9% normal saline and divided; one portion was immediately snap-frozen in liquid nitrogen and stored at –80°C for protein extraction, and the other portions were fixed in 10% formalin and embedded either in paraffin for histology analysis or in optimal cutting temperature (OCT) compound for immunofluorescence staining.

Animal Studies

Wild type (WT) (C57BL/6), homozygous *Nlrp3* knockout (*Nlrp3*$^{-/-}$; B6.129S6-*Nlrp3*^{tm1Bhk}/J), and *caspase-1* knockout (*Casp1*$^{-/-}$; B6N.129S2-*Casp1*^{tm1Flv}/J) mice were obtained from Jackson Laboratories (Bar Harbor, ME). We determined the sample size of mice for our experiments. Our preliminary data indicated that the overall incidence of AAD from all aortic segments examined was almost 80% in WT mice, 40% in *Nlrp3-/-* mice, and 11% in *Casp1-/-* mice. Thus, we estimated that 20 mice in each group were needed to show a statistically significant difference in the incidence of AAD (at α=0.05 with 80% power). However, to ensure sufficient aortic samples for biomechanical testing, histology, and protein analyses, we used higher numbers of mice in each group: 28 WT mice, 39 *Nlrp3-/-* mice, and 28 *Casp1-/-* mice. We randomized 4-week-old male mice to different treatment groups. In the challenged groups, we fed mice a high-fat diet (HFD) for 8 weeks and infused 2,000 ng/min/kg angiotensin II (AngII) (Sigma-Aldrich Corp., St. Louis, MO) during the last 4 weeks through an osmotic minipump (Model 2004; ALZA Scientific Products, Mountain View, CA). The HFD (Research Diets, Inc., D12108C, New Brunswick, NJ) contained 20% protein, 40% carbohydrate, 40% fat, and 1.25% cholesterol. Mice fed chow diet and infused with saline were used as unchallenged controls. For experiments with glyburide, the mice were challenged as described above and were given either glyburide (5 mg/kg) or water daily by oral gavage during the Ang II treatment period. Systolic blood pressure was measured weekly in conscious mice by using a noninvasive tail cuff BP-2000 system (Visitech Systems, Inc., Apex, NC). Glucose levels were also measured weekly. At the end of the 28-day saline or AngII infusion, mice were euthanized, and their aortas were irrigated with phosphate-buffered saline (PBS). Aortas were fixed in OCT compound for immunohistochemical and immunofluorescence staining or were snap-frozen for protein analysis.

Incidence of Aortic Aneurysm, Dissection, and Rupture

We measured the diameter of the ascending, arch, descending thoracic, suprarenal abdominal, and infrarenal aortic segments of the extracted aortas. In euthanized mice, we exposed and rinsed the aorta with cold PBS and removed the peri-aortic tissues. The aorta was excised and further cleaned and rinsed with cold PBS to remove any residual blood in the lumen. Images of the aorta were obtained using an Olympus SZX7 microscope at the magnification of 0.4X (scale

bar 2mm), and the diameters at each aortic segments were measured with DP2-BSW software (Olympus) by two independent observers who were blinded to the animal group. The mean diameter of the different regions was calculated and compared among the groups. For each aortic segment, aortic aneurysm in challenged WT or *Nlrp3-/-* mice was defined as an aortic diameter >1.5 times the mean aortic diameter the segment in unchallenged mice with the same genetic background. We defined aortic dissection as the presence of a tear in the aorta with separation within the media or in the media-adventitia boundary and the presence of a false lumen hematoma in an aortic cross section. Aortic rupture and premature death were documented.

Severity of AAD

The gross appearance of each aorta was assessed for severity of AAD formation according to a scheme inspired by the classification system described by Daugherty and colleagues[1]: normal aorta; dilated aorta without dissection (between 1.25 and 1.49 x normal aortic diameter); single aortic aneurysm (1.5x normal aortic diameter or greater) without dissection; aortic dissection, as indicated by intramural thrombus; multiple distinct aortic aneurysms (with or without dissection); and ruptured aorta. The aneurysmal tissue was evaluated independently by 2 observers blinded to the animal group. In cases of discrepancies, the observers discussed the cases and reached an agreement on the classification.

Aortic Contractility

We studied aortic contractility in aortas from challenged or unchallenged WT, *Nrlp3-/-* mice, and *Caspase1-/-* mice. In euthanized mice, the aorta was exposed and continuously perfused with ice-cold, specially formulated Krebs buffer; the descending aorta from T4 to T10 was excised, placed in ice-cold DMT PBS solution, stripped of periaortic tissue, and then segmented into 2-mm–long segments as indicated on a millimeter ruler. Harvested aortic segments in Krebs buffer (mmol/L) (119 Nacl, 4.7 KCl, 1$MgSO_4$, 1.2 KH_2PO_4, 24 $NaHCO_3$, 11 glucose, 2.5 $CaCl_2$, pH 7.4) were mounted onto metal stirrups of a wire myograph machine at 37°C. The buffer was equilibrated with carbogen (95% O_2, 5% CO_2) and refreshed every 15 minutes. The aortas were equilibrated for 45 minutes under minimal force to a resting point of 15mN. Contractile force was measured by using an isometric force transducer, and the data were acquired with Power Lab. We added 40 mM of KCl to the Krebs buffer to contract the aortic rings until the force that developed was constant for 2 contractions. Upon equilibration, we measured the tension after adding phenylephrine (10^-9 – 10^-5) or KCL (5 – 125 mM) and generated dose-response curves.

Aortic Biomechanical Properties

The strain-stress relationship of the aorta was determined by using pressure myography analysis (DMT-USA, Ann Arbor, MI). Descending thoracic aortic segments were excised and stretched to lengths representative of those found in vivo. The aortic segments were pressurized in 25-mmHg increments from 25 mmHg to 225 mmHg. We recorded the changes in the outer diameter in response to each pressure increment, determined the pressure-diameter relationship, and established the strain-stress relationship and the elasticity of the aorta.

Aortic Structure

Paraffin or OCT-embedded aortic sections were stained with hematoxylin and eosin and Verhoeff–van Gieson elastin staining (Sigma-Aldrich) according to the manufacturer's instructions. Four independent observers who were blinded to the animal group allocation examined 3 aortic sections per aorta from 8-10 mice per group. The extent of elastic fiber fragmentation was scored on a scale of 0 to 3 (grade 0 = none, grade 1 = minimal, grade 2= moderate, and grade 3 = severe).

Immunohistochemistry and Immunofluorescence Staining
Formalin-fixed, paraffin-embedded aortic sections were processed and analyzed as previously described.[2] Deparaffinized sections or OCT sections were incubated with antibody against SM22-22a (Abcam, Cambridge, MA), CD68 (Santa Cruz Biotechnology, Inc., Dallas, TX, sc 9139), NLRP3 (Cell Signaling Technology, Danvers, MA), ASC (Santa Cruz, sc22514), caspase-1 (Santa Cruz, sc56036), or connexin V (Cell Signaling Technology, Inc., Danvers, MA) at 4ºC overnight. The sections were washed with PBS and then incubated with secondary antibody conjugated to an Alexa Fluor dye (Alexa Fluor-488, -555, or -647; Thermo Fisher Scientific, Waltham, MA). The nuclei were stained with 0.2 g/ml 4', 6-diamidino-2-phenylindole dihydrochloride at room temperature for 3 min. Slides treated only with normal IgG were used as negative controls. The images were captured by using Image-Pro Plus V4.5 software. Fluorescence was detected by using a Leica SP5 confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). For quantification, we randomly selected 4-5 fields (40X magnification) per tissue section. The same exposure time was used for all samples. The mean signal intensity as normalized to the aortic area was compared among the groups.

Western Blot Analysis
Frozen human tissues were crushed and homogenized in total protein extraction buffer (Thermo Scientific). Homogenates were dissolved in sample buffer, separated by using Mini-TGX gels (4-20%; Bio-Rad Laboratories, Inc., Hercules, CA), and transferred to a PVDF membrane with the use of Bio-Rad Turbo transfer. The membranes were incubated for 1 h in blocking solution comprising Tris-buffered saline containing 5% nonfat dried milk and 0.5% Tween 20 and then incubated overnight with primary antibodies against the following proteins: NLRP3 (Cell Signaling), ASC (Santa Cruz, sc22514), caspase-1 (Santa Cruz, sc56036), myosin heavy chain (Santa Cruz), or tropomyosin (Santa Cruz, sc 28543). The blots were then washed with PBS with tween, incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Cell Signaling), and developed with Clarity Enhanced Chemiluminescence (ECL; Bio-Rad). The blots were exposed with HyBlot ES autoradiography film (Denville Scientific Inc., Holliston, MA) and quantified by using Image J Software (National Institutes of Health, Bethesda, MD). We confirmed equal protein loading by immunoblotting with HRP-conjugated b-actin antibody (Santa Cruz) or GAPDH antibody (Santa Cruz).

Cell Culture
Human aortic SMCs (Cell Applications, Inc., San Diego, CA) were cultured in Smooth Muscle Cell Growth Medium (Cell Applications) that contained 10% fetal bovine serum (FBS). Cells with fewer than 8 passages were used in the experiments. After the transfection of cells with NLRP3 siRNA (Thermo Fisher), ASC siRNA (Thermo Fisher), or caspase-1 siRNA (Thermo Fisher) for 24 hours, palmitic acid (PA) was added for an additional 24 to 48 hours.

Preparation of Palmitic Acid
We used saturated PA in this study. Briefly, PA was dissolved in ethanol at 200 mmol/L and then combined with 10% PA–free, low-endotoxin bovine serum albumin (BSA) at a final concentration of 1 to 5 mmol/L. The pH of all solutions was adjusted to 7.5, and the stock solutions were filter-sterilized and stored at –20ºC until further use. Control solutions containing ethanol and BSA were prepared similarly. Working solutions were prepared fresh by diluting the stock solution (1:10) in 5% FBS Smooth Muscle Cell Growth Medium (Cell Applications). All PA media contained 1% BSA; the PA:BSA ratio varied with the PA concentration.

Smooth Muscle Contraction Assay

SMC contraction was examined by determining the ability of SMCs to contract in collagen matrix. We used SMCs that had undergone fewer than 6-8 passages. Treated SMCs were collected, mixed with collagen gel matrix (Cell Biolabs, Inc., San Diego, CA), and incubated at 37ºC for SMC polymerization and tension development. The collagen gel–SMC matrix was then released from the well walls, and the diameter of the collagen matrix was measured every 30 min for 8 hours.

Co-immunoprecipitation Analysis

SMCs were lysed for 60 minutes in ice-cold extraction buffer containing 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 50 mM β-glycerophosphate, and a protease inhibitor mixture (Sigma-Aldrich). Cell lysates were precleared with IgG-conjugated protein A/G-agarose beads (Santa Cruz) at 4°C for 1 hour. Lysates were then incubated at 4°C overnight with protein A/G agarose beads conjugated to primary antibody against NLRP3 (Santa Cruz, sc68846), caspase-1 (Santa Cruz), tropomyosin (Santa Cruz), or myosin heavy chain (Santa Cruz). The beads were washed 4 times with PBS and once with PBS containing 0.5 M LiCl. The beads were then mixed with sample buffer, heated at 95°C for 10 min, and subjected to western blot analysis.

Caspase-1 Cleavage Assay

Recombinant tropomyosin (Novus Biologicals, LLC, Littleton, CO), or immunoprecipitated myosin heavy chain was mixed with recombinant caspase-1 (EMD Millipore) with or without caspase-1 inhibitor Z-VAD-FMK (Santa Cruz) at 37°C for 60 to 120 min. Samples were denatured with 1X sample buffer at 95°C for 10 min, and the proteins were separated by performing SDS polyacrylamide gel electrophoresis (4-15% SDS) followed by incubation with the appropriate antibody.

Statistical Analysis

All quantitative data are presented as the mean ± standard deviation. Data were analyzed by using SPSS software, version 11.0 (International Business Machines Corp., Armonk, NY). The normality of the data was examined by using the Kolmogorov-Smirnov test. Two-group comparison was performed by using independent *t* tests and the Mann-Whitney test, and multiple groups were compared by using one-way analysis of variance or the Kruskal-Wallis test, as appropriate. The General Linear Model (ANCOVA) was used to adjust for both categorical and continuous predictors with Bonferroni corrections. For all statistical analyses, 2-tailed probability values were used. A probability value of $P<0.05$ was considered significant.

Study Approval

The human tissue study was approved by the institutional review boards at Baylor College of Medicine, CHI St. Luke's Health - Baylor St. Luke's Medical Center, and Washington University in St Louis. Informed written consent was obtained from all individuals included in the study. All animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine in accordance with the guidelines of the National Institutes of Health.

References

1. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J Clin Invest. 2000:105:1605-1612.
2. Shen YH, Zhang L, Ren P, Nguyen MT, Zou S, Wu D, Wang XL, Coselli JS, LeMaire SA. AKT2 confers protection against aortic aneurysms and dissections. Circ Res. 2013:112:618-632.